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GRANULOCYTE KINETICS IN CIRRHOSIS

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GRANULOCYTE KINETICS

IN

CIRRHOSIS

Submitted in partial fulfillment
of the requirements for the degree of

Doctor of Medicine

to the

Department of Medicine
Yale University School of Medicine
New Haven, Connecticut
April 1, 1968

by

Philip L. Barry, A.B.
Holy Cross College, 1964

1-2-1972

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UNIT 10

NAME

DATE

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- 2. The second part of the story is very interesting.
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I would like to express my gratitude to M. S. Greenberg, M.D. of the Lemuel Shattuck Hospital in Boston, Mass., under whose aegis these studies were carried out. His guidance and cooperation were invaluable.

I would also like to thank my faculty advisor at Yale, Stuart C. Finch, M. D. for his many suggestions and remarkable forbearance during the course of this project.

Finally, congratulations and thanks are due to Ruth Cox for her expert typing and her success in interpreting the chaos of the initial hand-written drafts.

MEMORANDUM

I would like to express my appreciation to Mr. J. J. Connelley,
A.D. of the Bureau, for his kind invitation to attend the
whose name I have not yet received. His visit
and cooperation were invaluable.
I would also like to thank Mr. J. J. Connelley for his
kind invitation to attend the course on the subject
of "The Psychology of the Crime".
I am, Sir, very respectfully,
Yours very truly,
J. J. Connelley

PURPOSE

The frequency with which infection develops as a complication of cirrhosis indicates that the body's defenses against bacterial invasion are in some way compromised by this disease. In view of the recognized role of the granulocyte in the prevention and control of infection, the not uncommon finding of leukopenia in association with cirrhosis suggests that the vulnerability of the patient with cirrhosis is at least partly related to inadequacies in this particular defensive system.

As will be pointed out, there is usually no apparent deficiency in the production of granulocytes in this group of patients. Thus studies were undertaken to define the kinetics of granulocytes already in circulation. Cell disappearance rates, serum enzymes, and the capacity to evolve an inflammatory response provide useful information in this regard. Evaluation of these parameters in patients with cirrhosis will be discussed.

REVIEW OF LITERATURE

The association of cirrhosis, leukopenia, and infection has been known for many years. In 1929, King¹ reported that approximately one-third of a series of 100 patients with cirrhosis had less than 6000 leukocytes/mm³ in the peripheral blood: 28% of his afebrile patients and 32% of those with fever, for which no cause could be found other than cirrhosis, were leukopenic. Armas-Cruz et al.² reported

a leukopenia incidence of 13.1% in 183 patients with cirrhosis. Masina³ indicated that the "majority" of his patients had white counts between 4000-6500, with some as low as 3300.

The elevated incidence of infection in cirrhosis has been documented at autopsy by McCartney⁴, who found that 16% of his patients died of infection - in most cases of pneumonia or peritonitis. Evans and Gray⁵ reported in 1938 that 37% of 217 people with cirrhosis died of pneumonia. Ratnoff and Patek⁶ ascribed nearly one quarter of deaths in this type of patient to infection, including 10.3% due to pneumonia and 5.6% from non-tuberculous peritonitis. They cite other reports in which 10% of deaths were caused by tuberculosis, and 26% by pneumonia, peritonitis or erysipelas⁷, and in which the incidence of acute endocarditis was twice that of a non-cirrhotic population.⁸ Several reports⁹⁻¹² have noted the occurrence of bacteremia and peritonitis associated with enteric organisms, in the absence of a recognizable source of infection. Although decreased local resistance in the intestinal mucosa and reduced hepatic filtration of the absorbed organisms may account for many of these cases, as postulated by Conn¹¹, this explanation would seem less plausible in the cases of spontaneous peritonitis due to pneumococcal infection, as reported by Epstein¹³.

The mechanisms underlying the cytopenias of cirrhosis

1. The first thing I noticed when I stepped out of the plane was the cold air.

2. It felt like a giant hand was squeezing me, and I knew I was in for a long one.

3. The pilot told me that the weather was bad, but I didn't mind. I was here to see the sights.

4. I was told that the city was beautiful, and I was determined to see it all.

5. The first thing I noticed when I stepped out of the plane was the cold air.

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27. The pilot told me that the weather was bad, but I didn't mind. I was here to see the sights.

have been subject to considerable debate. It is widely recognized that the cirrhotic population is often nutritionally deficient. Yet the appearance of the bone marrow in these individuals is not infrequently normal or even hyperactive in terms of cellularity, with no morphologic abnormalities. This suggests that the low blood counts encountered clinically are probably not explainable simply on the basis of marrow depression secondary to poor intake of vitamins or other dietary essentials.

A plausible alternative explanation is that a physiologic mechanism of granulocyte removal is exaggerated in the disease. Several workers have described the normal processes. Vejlsens,¹⁴ Weisberger,¹⁵ Ambrus et al.,¹⁶⁻¹⁸ Leahy,¹⁹ Bierman et al.,^{20,21} and Kumar et al.,²² have discussed the importance of the liver, lung and spleen in this regard. The lung was found to remove damaged cells, probably on their first pass through the organ, and it is capable of maintaining the white count within certain limits by a process of margination and release of polymorphonuclear cells from the walls of capillaries and venules. It responds more rapidly than the liver or the spleen. However, the liver ultimately accumulates the largest proportion of experimentally infused cells. As noted by Barcroft,²³ the spleen, which had long been recognized as a "reservoir" of blood,^{24,25} that is outside

of the general circulation,²⁶⁻²⁸ also acts slowly. Normally, it accounts for the removal of fewer cells than either the lung or the liver.

Well before the semi-quantitative studies begun in 1950 by Weisberger and his contemporaries, attention had been drawn to the enlarged spleen as a site of pathological reduction of leukocyte concentration. Bauer²⁹ made the first recorded association between leukopenia and splenomegaly in 1899. Over the years it was recognized that several disorders frequently characterized by splenomegaly also had a significant incidence of leukopenia. Tuberculosis, malaria, kala-azar,³⁰ syphilis and splenic vein obstruction,³¹ as well as cirrhosis, are prominent in this category. In 1913, Osler³² attributed the leukopenia of Banti's "disease", first described in 1883,³³ to splenomegaly. Felty's syndrome was described in 1924,³⁴ but at that time the author did not establish a causal relationship between the leukopenia and splenomegaly.

In 1939, Wiseman and Doan³⁵ provided new evidence for the role of the enlarged spleen in the regulation of granulocyte concentration with the first of several reports³⁶⁻⁴² of primary splenic neutropenia, in which there was no abnormality other than an enlarged spleen to account for the depressed granulocyte counts.

These clinical findings gave substance to the concept of "hypersplenism", defined by Dameshek⁴³ as the combination

of splenomegaly, cytopenia(s) and normal or hypercellular bone marrow. Any of these factors could be absent in a specific case, but all should be correctable by splenectomy. In 1941, Dameshek⁴⁴ pointed out that there appeared to be a similarity among disorders representing the three groups of blood elements, all of which were dependent on splenic dysfunction: congenital hemolytic jaundice affecting erythrocytes, thrombocytopenic purpura due to platelet deficiency, and splenic neutropenia involving leukocytes.

In several publications, Dameshek^{43,45,46} maintained that in addition to its normal function in blood destruction, the spleen ordinarily exerts an inhibitory effect either on blood cell production or on release of mature cells from the bone marrow. He felt that this process was mediated by a humoral factor evolved by the spleen, and that the splenomegaly of hypersplenism caused an increased production of this substance and, secondarily, increased marrow inhibition and peripheral cytopenia.

In 1937, Lifshitz⁴⁷ observed that the leukocytosis expected after splenectomy in rats could be reduced by re-implanting splenic tissue into the animals. He concluded that the ectopic transplants were producing an endocrine-like substance suppressing the marrow. Palmer⁴⁸ agreed on the basis of similar studies.

An interesting paper supporting the humoral hypothesis was published in 1956 by Sen et al.⁴⁹ They reasoned

that if the spleen regulated granulocyte concentration by sequestration and destruction of the white cells within the spleen, removal of portions of the spleen should result in an increase in peripheral white count proportional to the amount of splenic tissue removed. Working with rats, they found that this did not occur. Twenty-five to fifty percent of the spleen was capable of maintaining normal levels of circulating granulocytes.

Opinions about the relationship of splenic size and degree of cytopenia in non-surgical circumstances varied. Dameshek's clinical impression was that there was, in general, a correlation.⁴⁵ Giblett⁵⁰ reached the same conclusion in a study of hemolytic anemia in rats made hypersplenic with methyl cellulose. Palmer,⁵¹ on the other hand, found no such relationship for granulocytes in the same animal model.

A different mechanism for hypersplenic leukopenia was popularized by Doan,⁵² who discounted the concept of marrow suppression, but felt that the cells were being removed from circulation at an accelerated rate. In his preliminary report of primary splenic neutropenia,³⁵ he reported extreme splenic clasmatocytosis, with excessive phagocytosis of granulocytes. Dameshek⁴³ and others^{42,53} did not find this, but several reports confirmed the observation.^{36,37,54} Doan⁵⁵ pointed out that supra-vital

staining of fresh material was a more effective way of demonstrating the phagocytosis than examination of fixed sections, and that failure to use this technique might account for some of the negative findings reported. Adding weight to Doan's hypothesis was the observation that blood granulocyte concentration was lowered considerably by passage through an enlarged spleen.⁵⁴ Techniques for following a particular population of granulocytes to their ultimate elimination from the circulating blood were not available when the controversy over hypersplenism was at its peak.

Considering the data available, Crosby³⁰ probably represented the majority opinion when he concluded, in 1962, that Doan was correct, i.e., that the leukopenia of hypersplenism is due to excessive sequestration and lysis of granulocytes by splenic phagocytes, and not to humoral depression of the bone marrow.

Although the basis for enlargement of the spleen is presumably different in primary and in secondary hypersplenic leukopenias, i.e., "idiopathic" in the former, and the result of portal hypertension of cirrhosis⁵⁶ or systemic disease in the latter, the organ is similar in morphology and function in each case, and the mechanism of the cytopenia is presumably common to both.

With the introduction of diisopropylfluorophosphate

in radioactive form (DFP³²) as a granulocyte label by Pollycove et al. in 1957,^{57,58} the opportunity arose for measurement of new parameters of granulocyte kinetics. Athens, Mauer, and their associates extensively evaluated this label and used it in an excellent series of studies of granulopoiesis and kinetics.⁵⁹⁻⁷² They determined that in normal man labeled granulocytes leave the total blood granulocyte pool in an exponential fashion with a mean half time disappearance ($T_{\frac{1}{2}}$) of 6.7 hours. Other workers, using less physiological or less direct techniques, have arrived at figures ranging from 6 to 24 hours.⁷³⁻⁷⁶

In 1964, Mauer and Krill⁷² used DFP³² to study patients with granulocytopenias of various etiologies. One of their patients (#5) had cirrhosis with a large spleen, persistent neutropenia, myeloid hyperplasia, and a $T_{\frac{1}{2}}$ of less than 30 minutes.

Serum lysozyme concentrations provide another guide to the rate of granulocyte destruction. Among the leukocytes, only the neutrophilic and monocytic elements contain significant quantities of this enzyme,⁷⁷⁻⁷⁸ and destruction of these cell types presumably accounts for most of the enzyme present in the serum.⁷⁹ It has been shown that there is a direct relationship between the serum lysozyme level and the number of circulating granulocytes in healthy individuals.⁸⁰

Furthermore, it appears that aberrations from the

normal relationship can be used to distinguish between defects in granulocyte production and destruction.⁸¹ Finch⁷⁹ has shown that the ratio of lysozyme to the total concentration of granulocytes and monocytes ("lysozyme index") is elevated in hypersplenism. Reed et al.⁸² have confirmed this in a group of patients with cirrhosis.

The significance of leukocytes in the inflammatory response is well recognized. Dutrochet⁸³ first noted in 1828 that "extravasation of white blood cells is the cardinal feature of inflammation".⁸⁴ Metchnikoff⁸⁵ observed the phenomenon of phagocytosis in 1883. Leber's⁸⁶ work in 1888 resulted in the concept of chemotaxis.

Since these early studies, a vast literature has appeared concerning the origins and modifications of the cells seen sequentially in inflammatory exudates and the mechanisms involved. Rebuck and Crowley⁸⁷ reviewed much of this work in 1955.

Many workers have implicated the granulocyte in the initiation of the inflammatory cycle. Menkin⁸⁸ postulated that "leukotaxine", a nitrogenous compound released by damaged tissue, caused increased capillary permeability and emigration of polymorphonuclear cells.

In 1954, Harris⁸⁹ felt that polypeptides produced by enzymatic digestion of protein at the site of injury were responsible for the initial emigration of granulocytes. Despite the difference in the ultimate histologic appearance

of infections caused by staphylococcus aureus and by tuberculosis, Harris found that the initial (granulocytic) phase was the same in each case. By 1960, he had concluded that chemotaxis depended on an interaction of polymorphonuclear cells and an altered vascular endothelium at the site of injury.⁹⁰

Hurley⁹¹⁻⁹³ believed that there was a serum factor that was activated by a tissue factor found to be particularly active in granulocytes. The activated compound permitted white cell emigration by alteration of the vascular walls.

A complete understanding of the basic process of cell migration is still not at hand. However, with the introduction of the cover-slip skin window technique by Rebuck⁹⁴ in 1947, a simple, direct method of studying the end result of the process became available. In a series of papers, Rebuck and his colleagues^{87,94-98} described qualitatively the sequential evolution of the inflammatory exudate under normal circumstances and in the presence of exogenous steroids.

A number of authors have used Rebuck's technique in a variety of clinical situations. In 1958, as part of the first study of any sort dealing specifically with the function of neutrophils in the inflammatory response, Page and Good⁴⁰ reported a case of cyclic neutropenia with

markedly depressed exudate production. In studies of leukemic patients with the cover-slip technique, Perillie and Finch⁹⁹ concluded that the quantity of cells exuded was depressed in the acute stage of the disease. Boggs¹⁰⁰ agreed, stating that the granulocytic character of the exudate seemed proportional to the number of mature polymorphonuclear cells and metamyelocytes in circulation.

Abnormal responses have also been demonstrated by this technique in patients with ulcerative colitis,¹⁰¹ allergy,¹⁰² interstitial cystitis,¹⁰³ cancer,¹⁰⁴ and psoriasis.¹⁰⁵

More realistic quantitation of the exudative process was attempted in Boggs,^{106,107} use of cantharidin-induced blisters. Others have further refined the technique by applying various types of chambers to the skin abrasion. In this way, Perillie and Finch¹⁰⁸ confirmed their earlier findings in leukemia. Brayton, Stokes and Louria¹⁰⁹ obtained normal results in patients with diabetes, uremia or cirrhosis, reduced counts in shock and acute alcoholism, and slightly elevated counts during major surgery with general anesthesia.

MATERIALS AND METHODS

Subjects: Studies were carried out on hospitalized patients, both male and female, ranging in age from 29 to 78. The diagnostic history and physical findings of cirrhosis were supported by liver function tests, and in many cases by liver biopsy and radiological evidence of esophageal varices. Patients with evidence of concomitant disease which could in itself contribute to a leukopenia were excluded from the studies. No patient showed depressed granulopoiesis on bone marrow examination. Most had granulocyte counts of less than 3500 per mm.³ Blood urea nitrogen values were normal. All had been on full hospital diet for at least two weeks and were assumed to be in good nutritional balance at the time of the studies.

Control subjects were hospital staff members and employees, and orthopedic or neurologic patients undergoing rehabilitation. None had evidence of liver or blood disorders.

The granulocyte disappearance and lysozyme studies, and the inflammatory response studies were done at different times and on different groups of patients.

DFP³² as a label: DFP³² inhibits esterase irreversibly. It has at various times been used to label erythrocytes and plasma,¹¹¹ platelets¹¹² and, as noted previously, leukocytes.

[illegible]

negative correlation between the number of years since graduation and

In their series of papers, Athens' group demonstrated that neutrophils were the only leukocytes to bind DFP³², that it was rapidly bound and was not eluted from the cell, and that binding had no effect on the viability of the cell. It was also noted that the isotope was rapidly excreted without reutilization after the initially labeled cell had been destroyed, and that in the dosage employed, there is no danger of radioactive or pharmacologic toxicity.

Granulocyte Labeling: Granulocytes were labeled and their subsequent survival in vivo was followed according to Mauer's method,⁶² with minor variations.

Approximately 400 ml of the patient's blood was drawn into ACD-A anticoagulated blood bags.* 0.45 mg. (0.9 μ c per ml of blood) of DFP^{32**} were added and gently mixed. The mixture was incubated for one hour at room temperature to allow for uptake of the label, and it was then reinfused into the donor within 15 minutes.

Collection of Samples: 20 ml of blood was drawn into 150 USP units of heparin sodium immediately after the end of the infusion (T₀) and at 0.25, 0.50, 0.75, 1, 2, 4, 6, and 24 hours. Part of each sample was removed for white counts and differentials.

*Fenwal blood pack unit #JA-2C

**New England Nuclear Corp.

Separation of Granulocytes: The white cell separation procedure was essentially that of Galbraith.¹¹⁴ 40 ml of dextran* was mixed with each blood sample. Red cells were allowed to sediment out for 30 minutes. The supernatant solution was then centrifuged at 130 x g. The resulting precipitate was washed in isotonic saline and spun at 55 x g to remove platelets. The remaining cells were suspended in 10 ml of 0.15 M NaAc buffer at pH 7.35. Red cells were lysed with 3% saponin. The suspension was then spun at 690 x g. The precipitate was washed in acetate buffer, and spun at 220 x g. Procedures were performed in an ice bath, using siliconized glassware. Centrifugations were carried out at 4° C. for 10 minutes.

Measurement of Radioactivity: A fine suspension of the final leukocyte button was made in 0.5-1.0 ml of absolute ethanol. This was pipetted onto weighed planchets and dried. The planchets were then reweighed to determine sample weight. A Geiger-Muller tube** was used for measuring radioactivity.

Lysozymes: Serum lysozyme concentrations were determined photometrically. The change in turbidity of Bacto-Lysozyme Substrate (Diffco) effected by the free enzyme in

*Clinical grade, Mol. wt. 200,000 - 300,000.
Nutritional Biochemical Corp.

**Nuclear Chicago, model D-47.

THEORY OF THE EARTH

1. The earth is a sphere of about 8000 miles in diameter.

2. The earth is composed of various layers.

3. The layers of the earth are the crust, the mantle, and the core.

4. The crust is the outermost layer of the earth.

5. The mantle is the layer between the crust and the core.

6. The core is the innermost layer of the earth.

7. The crust is about 10 miles thick.

8. The mantle is about 2000 miles thick.

9. The core is about 4000 miles thick.

10. The earth is composed of about 90% solid material.

11. The earth is composed of about 10% liquid material.

12. The earth is composed of about 1% gaseous material.

THEORY OF THE EARTH

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THEORY OF THE EARTH

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7. The crust is about 10 miles thick.

8. The mantle is about 2000 miles thick.

9. The core is about 4000 miles thick.

10. The earth is composed of about 90% solid material.

the serum was measured at 540 m μ on a Coleman Jr. Spectrophotometer.

Inflammatory Response: A Sykes-Moore tissue culture chamber¹¹⁵ was used to study cellular inflammatory response according to the modified Rebuck technique described by Southam and Levin.¹¹⁰ The lesion was standardized as much as possible by uniformly abrading the area within a circle 1.8 mm in diameter traced on the subject's forearm.

The chamber was held in place with adhesive tape and an Ace bandage. Leakage experienced in preliminary trials was eliminated by coating the bottom rim of the chamber with tincture of benzoin.

Approximately 1.0 ml of Gey's tissue culture medium was injected through the chamber's rubber gasket with 25 Ga. needles. 0.1 ml of diphtheria toxoid served as the antigenic stimulus.

The chamber was emptied by drawing 4 ml of fresh Gey's solution from one syringe to another through the chamber, and then with the chamber still in place, creating an air vent by removing the syringe from one needle. In this way, the remaining 1 ml of diluted cell suspension was drained into the collection syringe.

The total volume of fluid was recorded, and cell counts were done in a hemocytometer. In most cases, differential cell counts could be done on the sediment of the suspension, on the cover slip from the chamber, and

The author has indicated that the work is a preliminary study of the problem of the

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on touch preparations from the lesion itself.

RESULTS

I. Granulocyte Disappearance: The specific activity of each sample was calculated on the basis of radio-activity per milligram of dried granulocyte. The latter figure was approximated by adjusting the leukocyte sample weight with the differential count from the corresponding blood sample. Specific activity, expressed as a percentage of the initial sample ($T_0 = 100\%$), was plotted over time on semi-log paper, and the half-life was determined from the graph. These data appear in Table A.

The results of studies on 10 normal individuals are shown in Figure I. As can be seen in this graph and in Figure II (drawn by averaging the values of the individual studies), the general pattern in normal subjects is the expected straight line, indicative of exponential disappearance of the labeled cells. The mean half-life was 4.4 hours (1 S.D. = 2.1 hrs.).

Figures III and IV represent the individual and composite results, respectively, from studies on 12 patients with cirrhosis. This group manifests a different pattern. There is an initial rapid decrease in specific activity, followed by a second, slower phase similar to the normal disappearance pattern.

These data appear in Table 1.

4.4 hours (2.1 = 2.1 hrs.).

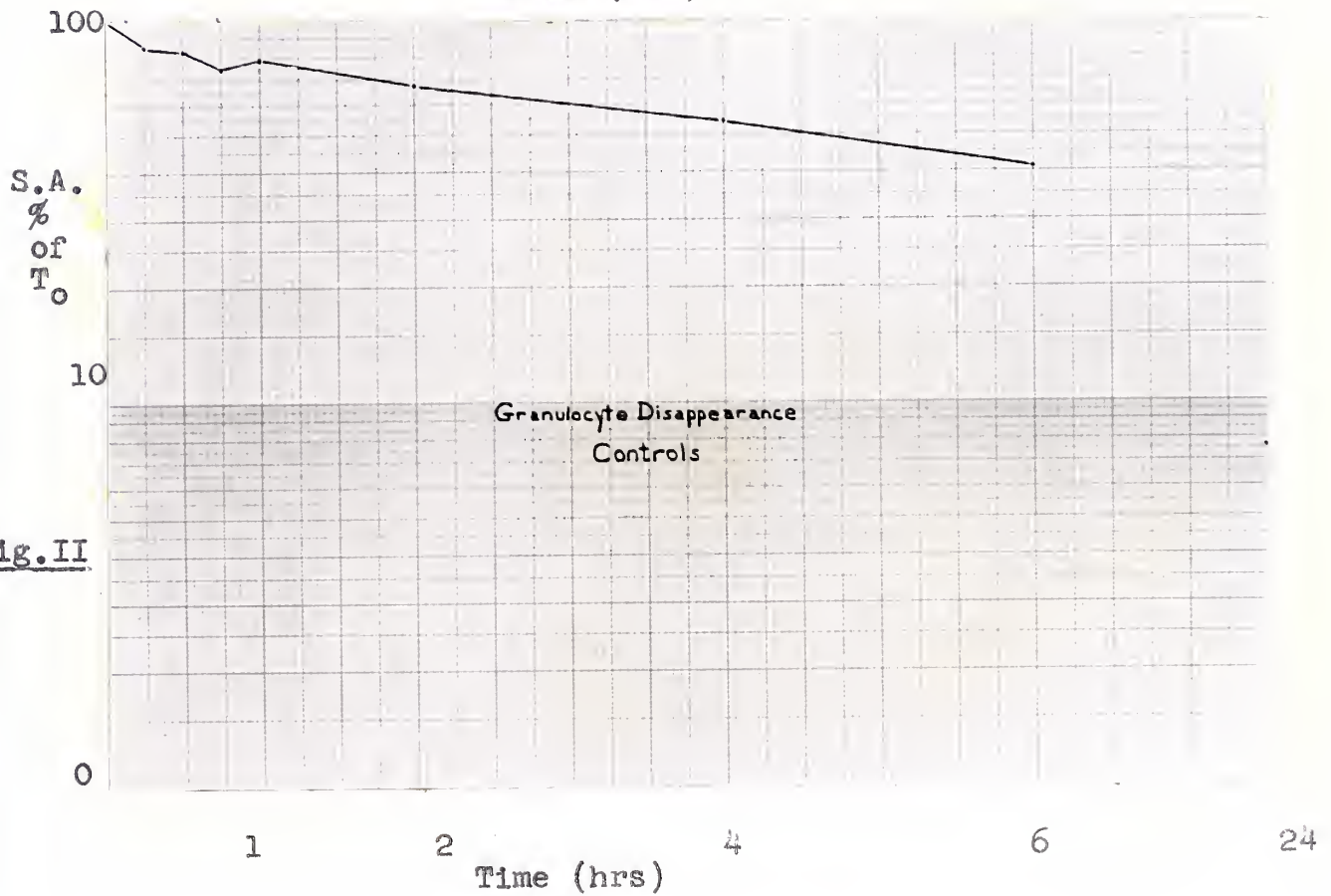
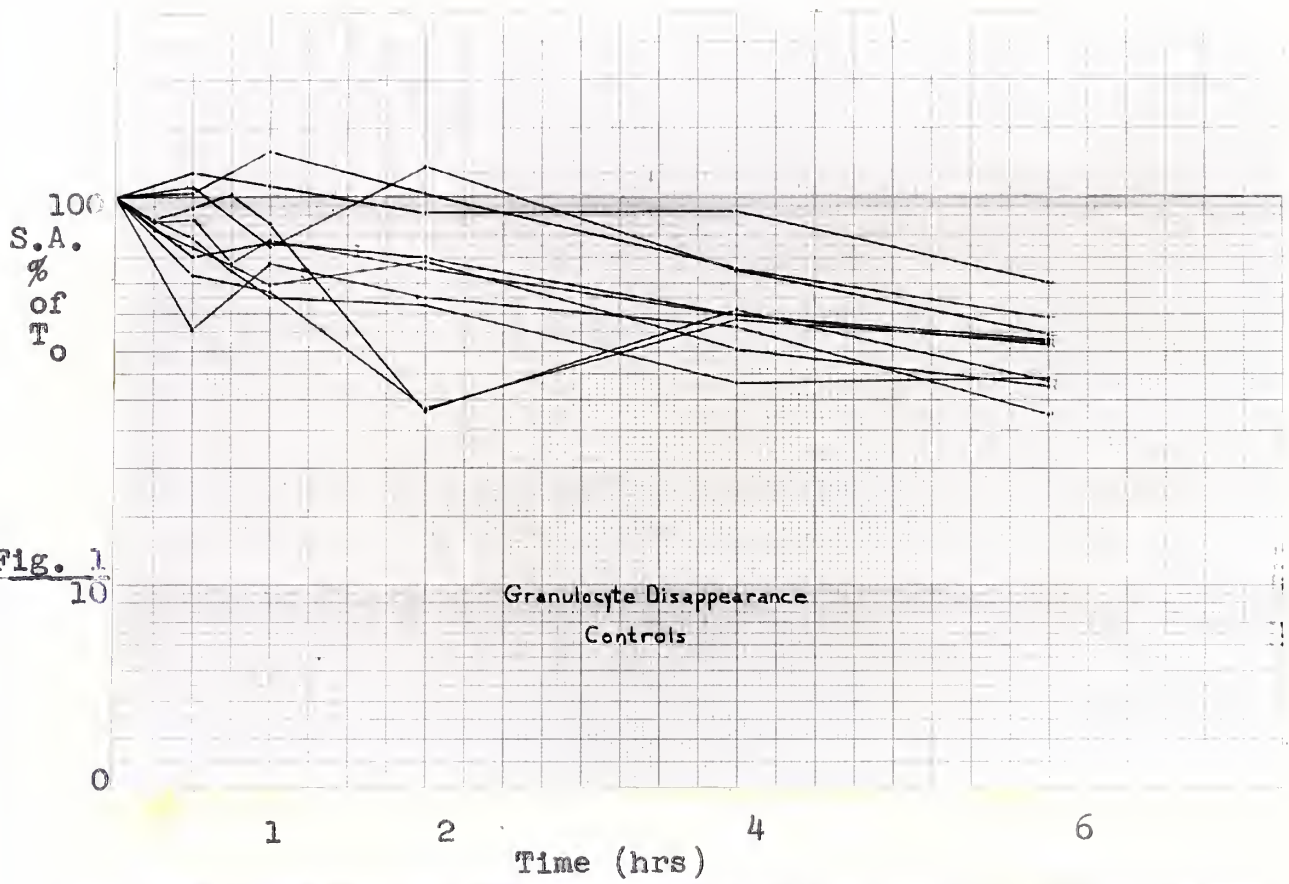
TABLE															
Subject	PMN/mm ³ (Baseline)	To	.25	.50	.75	1	1.5	Specific	Activity*		5	6	7	24 hrs.	T _{1/2}
								2	3	4					
Control															
1	5940	100	--	63.0	--	57.0	--	28.4	--	48.4	--	42.7	--	10.0	3.7
2	5760	100	87.7	78.7	64.6	55.1	--	52.9	--	33.1	--	34.3	--	9.8	2.0
3	3430	100	--	45.7	--	67.9	--	55.0	--	46.2	--	27.6	--	7.4	3.5
4	2550	100	82.2	74.2	66.8	59.1	--	68.5	--	40.6	--	32.5	--	7.0	2.7
5	6150	100	86.2	87.5	67.1	77.5	--	65.3	--	49.7	--	43.0	--	11.1	2.7
6	4570	100	--	101.5	--	131.0	--	101.0	--	65.2	--	49.2	--	--	3.8
7	5520	100	--	105.0	--	74.8	--	119.0	--	65.0	--	44.2	--	--	5.6
8	6210	100	--	115.0	--	106.0	--	91.0	--	92.4	--	60.6	--	10.3	6.1
9	4220	100	--	70.2	--	77.3	--	70.1	--	49.9	--	42.0	--	12.3	9.4
10	7190	100	87.3	94.1	101.0	85.8	--	28.2	--	51.3	--	33.8	--	8.0	4.0
Cirrhosis															
1	1840	100	--	57.8	--	48.0	--	42.5	--	37.0	--	27.2	--	9.7	0.9
2	1590	100	--	75.2	--	65.1	--	31.8	--	27.6	--	12.4	--	5.2	1.6
3	2310	100	--	66.0	--	52.3	--	37.0	--	32.9	--	28.7	--	6.0	1.1
4	2410	100	--	77.4	--	53.1	40.6	30.8	--	30.6	--	22.0	--	10.0	1.2
5	4090	100	--	--	--	50.6	--	--	39.9	--	31.1	--	20.6	5.8	1.2
6	3450	100	--	42.7	--	79.4	--	70.0	--	68.7	--	63.5	--	--	(8)
7	2660	100	--	50.8	40.7	34.8	--	33.9	--	36.2	--	29.4	--	9.5	0.5
8	483	100	--	39.0	--	13.1	5.5	11.6	--	11.2	--	9.1	--	3.8	0.3
9	5770	100	--	68.3	--	55.9	--	49.2	--	39.4	--	36.8	--	12.9	1.6
10	2030	100	--	16.4	--	24.1	--	18.4	--	21.6	--	24.9	--	8.8	0.3
11	6100	100	--	82.0	54.8	59.6	--	40.0	--	35.0	--	31.2	--	13.2	1.3
12	3100	100	--	70.1	63.4	58.8	--	50.5	--	31.4	--	20.6	--	2.4	1.9

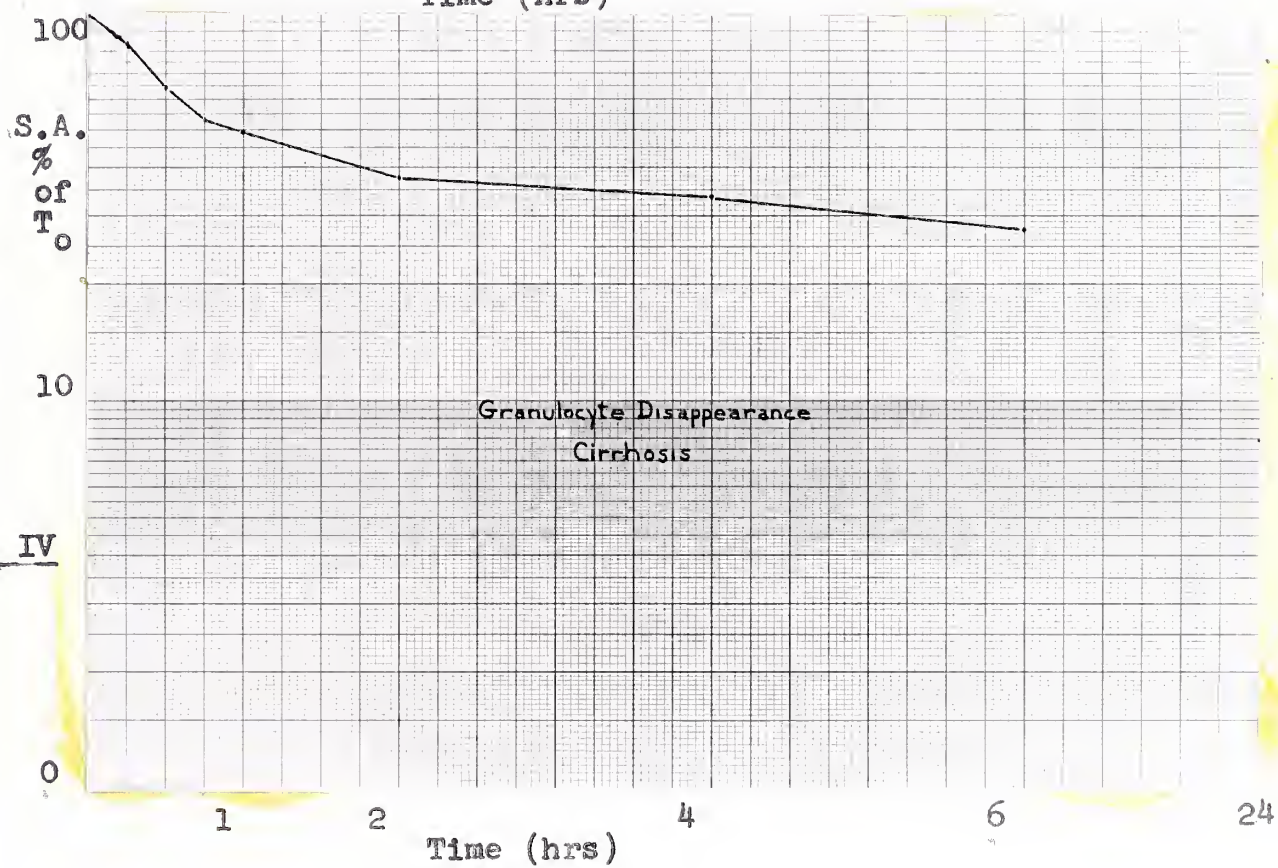
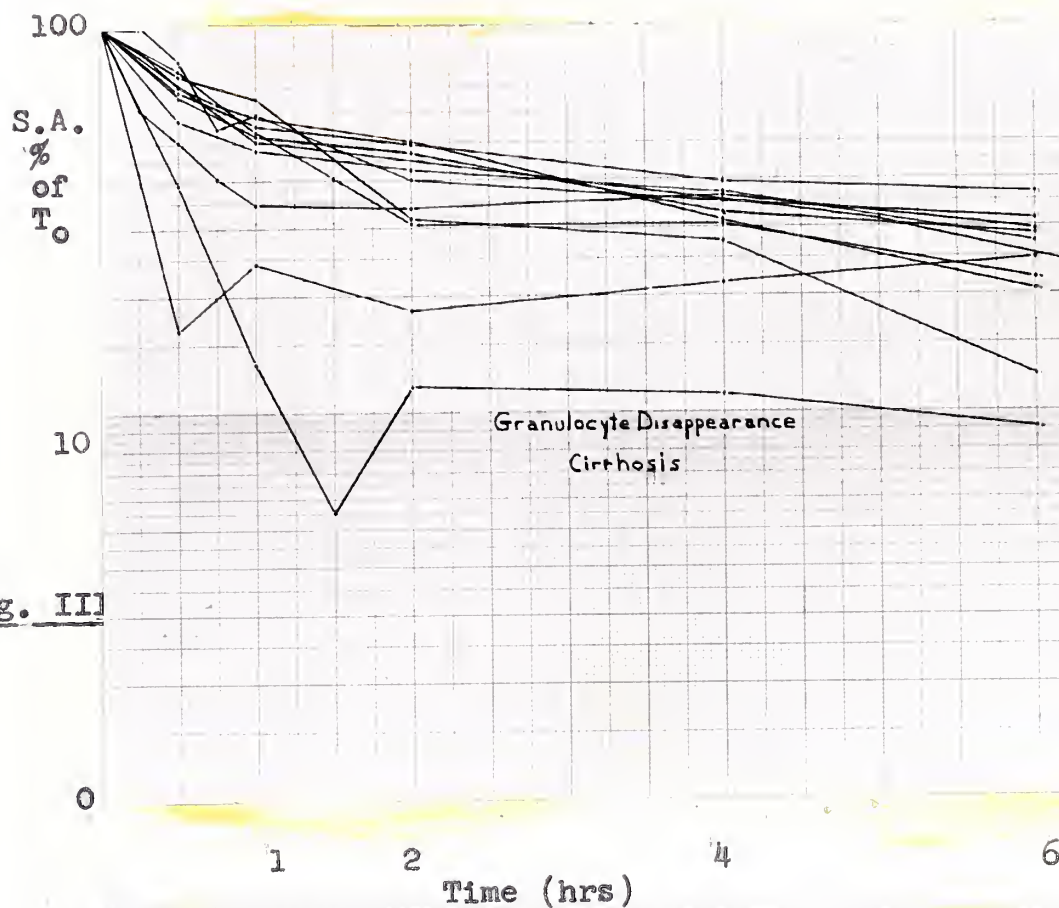
* Expressed in terms of cpm/mg of PMN, as % of To

Table A:

Granulocyte

Disappearance





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21

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21

01

01

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(cont) page 2

Eleven of the 12 studies had initial half-life values between 0.3 and 1.9 hours, with a mean $T_{\frac{1}{2}}$ of 1.08 hours (1 S.D. = 0.53 hrs.). In the other study, the specific activity of the 30 minute sample was 42.5% of T_0 , but the remaining samples indicate a $T_{\frac{1}{2}}$ well in excess of 8 hours. The gross deviation of these figures from the rest of the group suggests that unrecognized technical errors occurred, invalidating this particular study. The following tests show that error inherent in the procedures used is an unlikely explanation for this variation.

Reproducibility: In order to establish the reproducibility of the procedures used for leukocyte separation and radioactivity counting, 3 samples were drawn from the same bag of labeled blood. Each was processed in the usual fashion. At the final step each sample was divided and plated on two separate planchets. None of the resulting specific activities varied from the mean by more than 7.7%.

Purity of Leukocyte Separation: To rule out contamination of the separated white cell preparations, smears made of randomly chosen specimens were carefully examined for erythrocytes and platelets, which do bind DFP and could influence the radioactive counting as well as the weight of the specimen. No such elements were found. There were some areas of clumped white cells, and

small amounts of proteinaceous material (also capable of being labeled by DFP), but the quantities were minimal and probably of no significance.

Granulocyte Viability: The disappearance curves obtained in the patients with cirrhosis resemble the patterns seen when damaged cells are infused. To rule out the possibility that these patients' granulocytes are unusually susceptible to in vitro damage during the labeling procedure, two functions characteristic of the living granulocyte¹¹⁶ were compared in routinely labeled blood from normal and cirrhotic sources.

A. Phagocytosis: Aliquots of each type of blood were mixed with a suspension of heat killed, coagulase-positive staphylococcus aureus from a 24 hour broth culture. After incubation for 1 hour at 37° C smears were made and Gram stained. The percentage of phagocytizing granulocytes, and the number of organisms per phagocyte were recorded, with correction for "background" organisms.

B. Selective Permeability of Cell Wall: It is well recognized that living granulocytes resist penetration by several dyes, while dead cells are susceptible to the same dyes. On this basis, uptake of trypan blue (1:1000 dilution) was taken as evidence of non-viability. Labeled blood was mixed with equal volumes of dye, and thin wet mounts were prepared. The percentage of viable

cells was noted.

Results:	Normal	Cirrhosis
A. Phagocytosis: (% phagocytes in 300 PMN's)	93.7	92.4
# organisms per phagocyte	47.3 1 S.D.=9.7	44.8 1 S.D.=8.5
B. Dye Uptake: % unstained PMN's (300 cells)	94.0	95.4

Conclusion: The labeling procedure had no differential effect on the viability of the normal and cirrhotic subjects' granulocytes.

II. Lysozymes: Table B shows the serum lysozyme concentrations and the calculated lysozyme indices for each subject.

Subject	1	WBC	%PMN	PMN/mm ³	Lysozyme μg/ml	Lysozyme Index
Control						
	1	8,850	67	5,940	46.0	7.75
	2	8,350	69	5,760	47.0	8.16
	3	4,900	70	3,430	36.0	10.50
	4	5,000	51	2,550	55.0	21.60
	5	9,450	65	6,150	41.0	6.67
	6	5,500	83	4,570	55.0	11.80
	7	8,000	69	5,520	60.0	10.90
	8	8,750	71	6,210	38.0	6.12
	9	6,700	63	4,220	52.0	12.30
	10	10,550	68	7,190	60.0	8.35
	mean	7,605	67.6	5,154	49.0	10.4
	1 S.D.			1 380	8.3	5.06
Cirrhosis						
	1	3,400	54	1,840	46.0	25.0
	2	2,950	54	1,590	83.0	52.2
	3	4,050	57	2,310	52.0	22.6
	4	3,500	69	2,410	43.0	17.8
	5	6,200	66	4,090	42.0	10.3
	6	6,900	50	3,450	58.0	16.8
	7	4,100	65	2,660	66.0	24.8
	8	1,050	46	483	37.0	76.5
	9	7,500	77	5,770	89.0	15.4
	10	2,900	70	2,030	80.0	39.4
	11	9,100	67	6,100	67.0	11.0
	12	5,000	62	3,100	40.0	12.9
	mean	4,720	61.4	2,990	58.5	27.0
	1 S.D.			1 650	18.3	19.8
				p < 0.05	p > 0.05	p < 0.05

Table B: Lysozymes

10/10/1910

1. 10/10/1910
2. 10/10/1910
3. 10/10/1910

4. 10/10/1910
5. 10/10/1910

6. 10/10/1910
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13. 10/10/1910

14. 10/10/1910
15. 10/10/1910
16. 10/10/1910

17. 10/10/1910

18.	10/10/1910	19.	10/10/1910	20.	10/10/1910	21.	10/10/1910	22.	10/10/1910	23.	10/10/1910	24.	10/10/1910	25.	10/10/1910	26.	10/10/1910	27.	10/10/1910	28.	10/10/1910	29.	10/10/1910	30.	10/10/1910	31.	10/10/1910	32.	10/10/1910	33.	10/10/1910	34.	10/10/1910	35.	10/10/1910	36.	10/10/1910	37.	10/10/1910	38.	10/10/1910	39.	10/10/1910	40.	10/10/1910	41.	10/10/1910	42.	10/10/1910	43.	10/10/1910	44.	10/10/1910	45.	10/10/1910	46.	10/10/1910	47.	10/10/1910	48.	10/10/1910	49.	10/10/1910	50.	10/10/1910	51.	10/10/1910	52.	10/10/1910	53.	10/10/1910	54.	10/10/1910	55.	10/10/1910	56.	10/10/1910	57.	10/10/1910	58.	10/10/1910	59.	10/10/1910	60.	10/10/1910	61.	10/10/1910	62.	10/10/1910	63.	10/10/1910	64.	10/10/1910	65.	10/10/1910	66.	10/10/1910	67.	10/10/1910	68.	10/10/1910	69.	10/10/1910	70.	10/10/1910	71.	10/10/1910	72.	10/10/1910	73.	10/10/1910	74.	10/10/1910	75.	10/10/1910	76.	10/10/1910	77.	10/10/1910	78.	10/10/1910	79.	10/10/1910	80.	10/10/1910	81.	10/10/1910	82.	10/10/1910	83.	10/10/1910	84.	10/10/1910	85.	10/10/1910	86.	10/10/1910	87.	10/10/1910	88.	10/10/1910	89.	10/10/1910	90.	10/10/1910	91.	10/10/1910	92.	10/10/1910	93.	10/10/1910	94.	10/10/1910	95.	10/10/1910	96.	10/10/1910	97.	10/10/1910	98.	10/10/1910	99.	10/10/1910	100.	10/10/1910
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There is wide variation within each group. The raw lysozyme levels do not permit differentiation of the two populations.

However, the lysozyme index takes into account the fact that the serum lysozyme level is, in general, related to a smaller concentration of enzyme-releasing cells in the patients with cirrhosis. Using these adjusted values, a significant difference is demonstrated between the two populations sampled.

III. Inflammatory Response: Studies with the skin window demonstrated that in the early phases of the inflammatory response, the individuals with cirrhosis transferred fewer polymorphonuclear cells into the exudate than did control subjects. Figure V shows a relatively smooth rate of exudation by each group of subjects for at least the first eight hours. The normal subjects develop the cellular exudate five to six times faster than the cirrhotic group during this period.

It is apparent from Table C that an individual subject does not necessarily evolve the exudate in a smooth fashion, and that there is considerable variation from one subject to another. However, the trend seems clear, and the difference between groups is significant ($p < 0.05$) for the first 6 hours. At later stages in the inflammation, the scattering of values in the small groups studied yielded means that were not statistically separable.

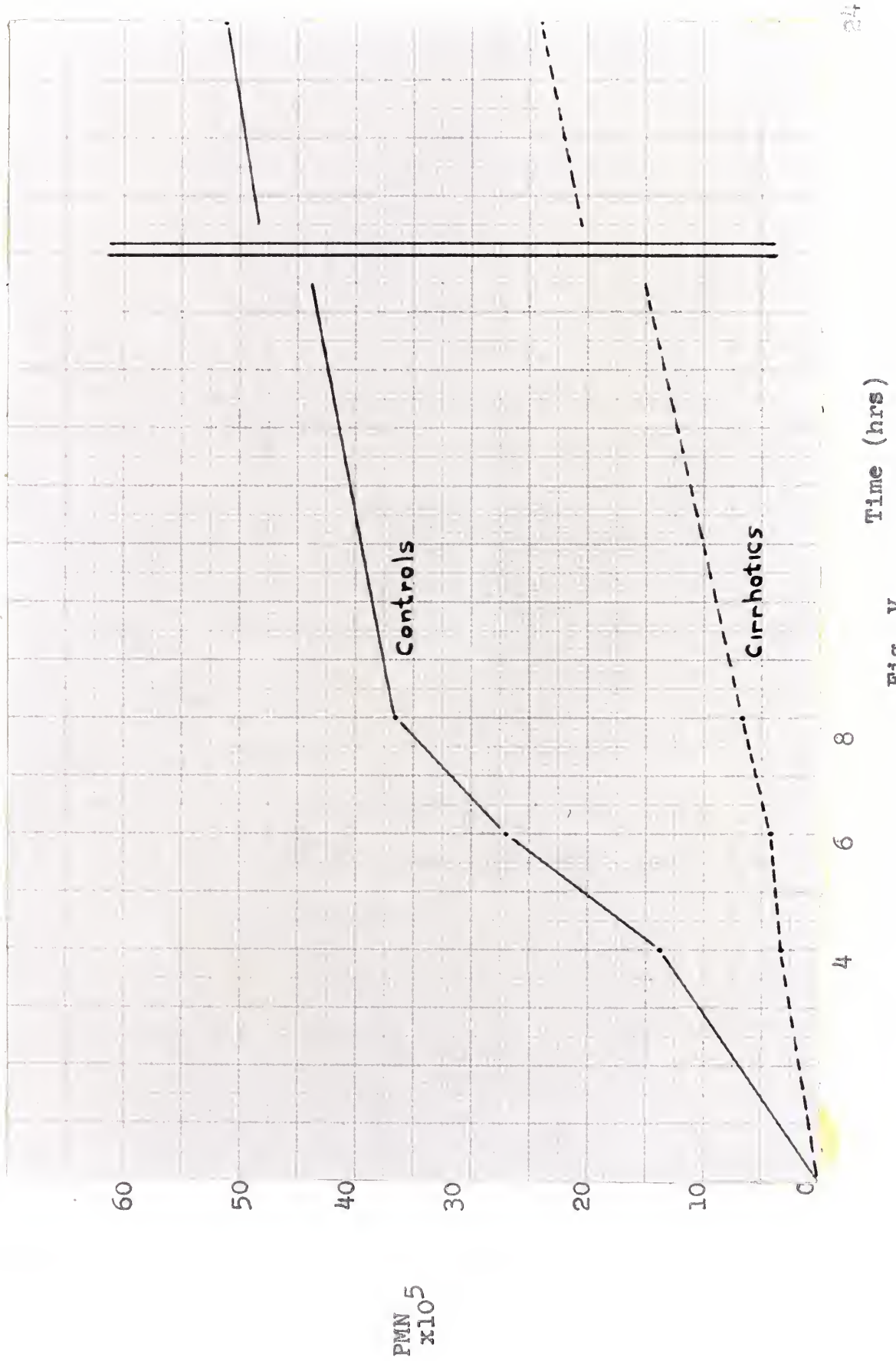


Fig. V

Subject	WBC	Time (hrs)				
		2	4	6	8	24
Control 1	1 9030		7.97	24.2	58.7	-
	2 8700		18.00	51.3	50.2	-
	3 4670		10.6	-	-	-
	4 5550		19.4	20.1	-	-
	5 4850		24.0	17.2	25.5	53.5
	6 6450		-	66.8	-	-
	7 4310		1.25	1.23	-	1.06
	8 6930		14.6	23.9	-	132.0
	9 3110		14.5	11.4	12.1	18.6
Cirrhosis	1 1410		2.99	2.95	4.04	-
	2 2860		4.75	4.74	4.90	37.10
	3 840		1.37	2.28	2.54	8.35
	4 3420		3.35	6.39	7.39	24.90
	5 2575		3.50	5.65	6.29	25.90
	6 3400		2.02	3.44	14.80	-

Table C: Total Number of PMN ($\times 10^5$) in Inflammatory Exudate

Table 1: Data for Figure 1

Year	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100																																																																																
Population	100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	240	245	250	255	260	265	270	275	280	285	290	295	300	305	310	315	320	325	330	335	340	345	350	355	360	365	370	375	380	385	390	395	400	405	410	415	420	425	430	435	440	445	450	455	460	465	470	475	480	485	490	495	500	505	510	515	520	525	530	535	540	545	550	555	560	565	570	575	580	585	590	595	600	605	610	615	620	625	630	635	640	645	650	655	660	665	670	675	680	685	690	695	700	705	710	715	720	725	730	735	740	745	750	755	760	765	770	775	780	785	790	795	800	805	810	815	820	825	830	835	840	845	850	855	860	865	870	875	880	885	890	895	900	905	910	915	920	925	930	935	940	945	950	955	960	965	970	975	980	985	990	995	1000										
GDP	100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	240	245	250	255	260	265	270	275	280	285	290	295	300	305	310	315	320	325	330	335	340	345	350	355	360	365	370	375	380	385	390	395	400	405	410	415	420	425	430	435	440	445	450	455	460	465	470	475	480	485	490	495	500	505	510	515	520	525	530	535	540	545	550	555	560	565	570	575	580	585	590	595	600	605	610	615	620	625	630	635	640	645	650	655	660	665	670	675	680	685	690	695	700	705	710	715	720	725	730	735	740	745	750	755	760	765	770	775	780	785	790	795	800	805	810	815	820	825	830	835	840	845	850	855	860	865	870	875	880	885	890	895	900	905	910	915	920	925	930	935	940	945	950	955	960	965	970	975	980	985	990	995	1000										
Unemployment	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0	11.5	12.0	12.5	13.0	13.5	14.0	14.5	15.0	15.5	16.0	16.5	17.0	17.5	18.0	18.5	19.0	19.5	20.0	20.5	21.0	21.5	22.0	22.5	23.0	23.5	24.0	24.5	25.0	25.5	26.0	26.5	27.0	27.5	28.0	28.5	29.0	29.5	30.0	30.5	31.0	31.5	32.0	32.5	33.0	33.5	34.0	34.5	35.0	35.5	36.0	36.5	37.0	37.5	38.0	38.5	39.0	39.5	40.0	40.5	41.0	41.5	42.0	42.5	43.0	43.5	44.0	44.5	45.0	45.5	46.0	46.5	47.0	47.5	48.0	48.5	49.0	49.5	50.0	50.5	51.0	51.5	52.0	52.5	53.0	53.5	54.0	54.5	55.0	55.5	56.0	56.5	57.0	57.5	58.0	58.5	59.0	59.5	60.0	60.5	61.0	61.5	62.0	62.5	63.0	63.5	64.0	64.5	65.0	65.5	66.0	66.5	67.0	67.5	68.0	68.5	69.0	69.5	70.0	70.5	71.0	71.5	72.0	72.5	73.0	73.5	74.0	74.5	75.0	75.5	76.0	76.5	77.0	77.5	78.0	78.5	79.0	79.5	80.0	80.5	81.0	81.5	82.0	82.5	83.0	83.5	84.0	84.5	85.0	85.5	86.0	86.5	87.0	87.5	88.0	88.5	89.0	89.5	90.0	90.5	91.0	91.5	92.0	92.5	93.0	93.5	94.0	94.5	95.0	95.5	96.0	96.5	97.0	97.5	98.0	98.5	99.0	99.5	100.0

Table 1: Data for Figure 1. The table shows the population, GDP, and unemployment rate for the years 1990 to 2100. The population is projected to increase from 100 in 1990 to 1000 in 2100. The GDP is projected to increase from 100 in 1990 to 1000 in 2100. The unemployment rate is projected to increase from 5.0% in 1990 to 100.0% in 2100.

DISCUSSION

Although the following comments are based on results that are statistically significant, in some instances the degree of significance was such that "p" was barely less than 0.05 because of fairly wide scattering of results from the small groups studied. Thus it is possible that, with data from larger groups, the results and consequently the conclusions could be materially altered. However, trends do emerge from the data at hand, and the results obtained will be discussed in these terms.

The granulocyte survival studies presented have suggested that infused, labeled granulocytes are removed from the circulation of the patient with cirrhosis more rapidly than from the individual without this disease. In seeking an explanation, one could look to an alteration in the relationship between the marginal and the circulating granulocyte pools. Athens et al.⁶³ discovered that in healthy adults these pools were of approximately equal size. The splenomegaly of the subjects in the present studies* certainly favors an increase in the size of the marginal pool, but the equilibration between the two pools is so rapid (i.e. complete by the end of the infusion) that it would not affect the slope of the disappearance curve in a manner that could explain the results obtained.

* 10 of the 12 cirrhotic subjects studied had palpable spleens, ranging from the left costal margins to 12 cm below the margin.

The fate of the circulating cells has been discussed in a number of papers,^{64, 70, 74, 119-121} which conclude that the extravasated granulocytes do not return to the circulation after migrating from it.

Noting that the disappearance rate of granulocytes cross-transfused from normal rats into irradiated, leukopenic rats was greater than normal, Hollingsworth and Finch¹²² suggested that the extra-vascular portion of the total body granulocyte pool (TBGP) was depleted to a significant extent, and that the infused cells were rapidly utilized to replenish this portion of the TBGP.

Osgood¹²³ has referred to the granulocytes as cells in transit from sites of manufacture to sites of extra-vascular defensive activity or loss, rather than as blood cells, per se. He points out that the circulating cells actually comprise a very small fraction of the TBGP. Thus when the larger extra-vascular portion of the TBGP is even minimally depleted, relatively large, rapid alterations in the peripheral count of infused cells could be expected.

In the chronic leukopenia frequently seen in patients with cirrhosis, such a mechanism would achieve greater significance in relation to the normal homeostatic functions of the lung and the liver in the regulation of granulocyte concentration.

The above is the first of the three parts of the report, which is a summary of the results of the investigation. The second part is a description of the methods used, and the third part is a discussion of the results. The first part is divided into two sections, the first of which is a description of the material and the second is a description of the results. The second part is divided into two sections, the first of which is a description of the methods used and the second is a description of the results. The third part is divided into two sections, the first of which is a discussion of the results and the second is a conclusion. The first part is divided into two sections, the first of which is a description of the material and the second is a description of the results. The second part is divided into two sections, the first of which is a description of the methods used and the second is a description of the results. The third part is divided into two sections, the first of which is a discussion of the results and the second is a conclusion.

Nevertheless, in the presence of cirrhosis, the spleen emerges as the dominant object of consideration. If one assumes perfectly normal activity in each unit mass of splenic tissue in such an individual who has splenomegaly, the increased mass of tissue would account for increased destruction of granulocytes. Yet, as Dameshek noted in his definition of hypersplenism, splenomegaly may be absent. However, the presence of leukopenia even without enlargement of the organ is understandable in view of the observations referred to previously which document an increased degree of splenic clasmatocytosis and phagocytosis of granulocytes in hypersplenism. Portal hypertension with secondary stasis of splenic blood flow, and auto-immune sensitization of granulocytes (in the fashion of platelets in idiopathic thrombocytopenic purpura and erythrocytes in acquired hemolytic anemia) are probably both factors in this process; their relative contributions cannot be ascertained.

The discussion above deals primarily with the initial, rapid phase of the cirrhotic patient's granulocyte disappearance curve. As was seen in Figure IV, the slope of the curve in cirrhosis flattens out considerably between 1 and 2 hours, in contrast to the continuous straight line of the normal graph (Figure II). Since it is unlikely that the body loses its affinity for the

cells, or that the granulocytes become more resistant to removal, a different compensatory mechanism must be sought.

Again, the abnormal spleen appears to provide an explanation. When the spleen is hypertrophied, a disproportionate fraction of the cardiac output enters and circulates through the sluggish vascular channels of the organ. It is not unreasonable to assume that many of the infused cells are rather rapidly sequestered by the hyperfunctional organ within the first few circulations through the systems, but that many of the cells of the infused "bolus" require a relatively long time to be flushed through the greater mass of splenic tissue and out into the peripheral circulation, thereby prolonging the later stages of the removal process.

Suggestive, but in no way conclusive information which supports this hypothesis was obtained in an animal study done in conjunction with the human projects described in this paper. A series of granulocyte survival studies on rats made hypersplenic with methylcellulose was carried out. Although the studies were not completed for technical reasons, preliminary data indicated that granulocytes in normal rats have a $T_{\frac{1}{2}}$ of approximately one hour. In the methylcellulose animals, whose spleens were 8 times heavier than control organs, there was no detectable radioactivity in the granulocytes separated from the 3 to 20 minute blood samples. However, between 45 and 180

minutes, measurable amounts of labeled cells were again in circulation.

Palmer et al.⁵¹ and Teoh¹²⁴ demonstrated that the cytopenias seen in this animal model were attributable specifically to the spleen, since methylcellulose administered to previously splenectomized animals failed to produce these changes. Thus, since the hypertrophy of the spleen is the only difference of functional significance between the groups of rats used in the survival time experiments, it is reasonable to speculate that this alteration is responsible for the apparent transient sequestration of leukocytes that was observed.

Although sequestration appears to have an exaggerated role in the granulocyte kinetics of cirrhosis, the prolongation of the time spent by the granulocyte in the spleen would also increase the likelihood that the cell would fall prey to the normal granulocytolytic mechanisms of the spleen. The lysozyme indices obtained in the present studies support the histologic findings of increased destruction as an additional explanation for the unusual rapidity of granulocyte disappearance in cirrhosis, and for the consequent leukopenia.

The full importance of the granulocytokinetic abnormalities in cirrhosis could best be evaluated by long-term prospective comparison of the incidence and severity of infection in groups of cirrhotic and of healthy

people. In lieu of this, the capacity to produce an inflammatory response serves as a roughly quantitative guide in the assessment of expectable susceptibility.

The data presented indicates that granulocytes emigrate poorly in the presence of cirrhosis. The differential counts and the cell morphology from the two groups studied were roughly comparable at given time intervals, but the total number of extravasated cells was markedly reduced in the early samples from patients with cirrhosis. Although the two populations are significantly different in this regard and in terms of the peripheral granulocyte concentration, no correlation could be made between these two parameters within each group.

The reduction of the inflammatory response in neutropenic individuals seen here is in agreement with the findings of Libansky¹²⁵ but conflicts with the preliminary data reported by Brayton et al.¹⁰⁹ with regard to cirrhosis. Yet, in view of the high incidence of infection, it is not surprising to find depression of the inflammatory response. It is possible that the rapidity with which granulocytes are removed from circulation, and apparently destroyed, may in some way reduce the availability of the granulocytes to the site of inflammation. Qualitative alterations in the chemotactic mechanisms seem an unlikely alternative explanation.

Inspection of the data given in Tables A, B and C gives rise to one additional observation. Although $T_{1/2}$,

lysozyme indices and inflammatory response are all altered in cirrhosis, the value for $T_{\frac{1}{2}}$ appears to be the one most consistently correlated with the clinical classification of the subject as "cirrhotic" or "normal".

SUMMARY

In studies of patients with cirrhosis, it has been shown that labeled granulocytes are removed from circulation at an abnormally high rate. Increased sequestration and destruction by the spleen, and extravascular loss probably contribute to this phenomenon. Lysozyme indices supported the suggestion of increased destruction as a factor in the leukopenia associated with this hypersplenic disease. Depressed inflammatory responses indicated that these factors may be of functional significance. Of the three parameters of granulocyte kinetics investigated, the rate of cell disappearance from the peripheral blood has the best correlation with the clinical picture.

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1. Introduction. The purpose of this study is to investigate the effects of the proposed changes on the system. The study is organized as follows: Chapter 2 describes the current system, Chapter 3 describes the proposed changes, Chapter 4 describes the results of the study, and Chapter 5 concludes the study.
2. Background. The current system is a legacy system that has been in use for many years. It is a complex system with many modules and a large number of users. The proposed changes are intended to improve the system's performance and reduce its maintenance costs.
3. Methodology. The study uses a combination of qualitative and quantitative methods. Qualitative methods include interviews with system users and experts, and a review of the system's documentation. Quantitative methods include a performance analysis of the current system and a simulation of the proposed changes.
4. Results. The results of the study show that the proposed changes will improve the system's performance and reduce its maintenance costs. The performance analysis shows that the proposed changes will reduce the system's response time by 20% and its CPU usage by 15%. The simulation shows that the proposed changes will reduce the system's maintenance costs by 10%.
5. Conclusion. The study concludes that the proposed changes are a good idea and should be implemented. The study also identifies some areas for further research, such as the effects of the proposed changes on the system's security and its compatibility with other systems.
6. References. The study references a number of books, articles, and reports. The references are listed in the following table:
7. Appendix. The study includes an appendix that contains the system's architecture diagram and a list of the system's modules.
8. Index. The study includes an index that lists the page numbers for each section of the study.
9. Summary. The study is a comprehensive study of the proposed changes to the system. It provides a detailed analysis of the current system and the proposed changes, and it provides a clear and concise summary of the results of the study.
10. Conclusion. The study concludes that the proposed changes are a good idea and should be implemented. The study also identifies some areas for further research, such as the effects of the proposed changes on the system's security and its compatibility with other systems.

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